REMARKS

It is respectfully requested that the above amendments be entered pursuant to the provisions of 37 C.F.R. §1.116(b); that this application be reconsidered in view of the above amendments and the following remarks; and that all of the claims remaining in this application be allowed.

Interview

The undersigned wishes to thank Examiner Meller for the courtesies extended to himself and Mr. Robert Hirons (assignee's representative) during the interview conducted for this application on December 16, 2003. The Interview Summary provided during this interview accurately reflects the discussions held which are elaborated upon below.

Amendments

Applicants have requested that Claim 19 be amended under the provisions of 37 C.F.R. §1.116(b) to more accurately reflect their invention. Specifically, Applicants have requested that Claim 19 be amended to be solely directed to apoptotic conditions (as opposed to necrotic conditions) and then to recite that the claimed method is directed to patients having or suspected of having a neurological medical disorder mediated by accelerated rates of apoptosis. Support for these amendments is found in Applicants' specification at, for example, page 6, lines 13-23, which recite neurological conditions are mediated by accelerated rates of apoptosis.

Claim 19 was further amended to delete reference to tissues and organs as this is inconsistent at worst and confusing at best with the treatment of neurological disorders.

Claim 23 was amended to correct an obvious typographical error and Claim 24 was amended to correspond to previously presented Claim 6.

Insofar as these amendments either conform with matters of form required by the USPTO and/or place this claim in better form for appeal, entry of these amendments under the provisions of 37 C.F.R. §1.116(b) is appropriate. Entry of these amendments is requested.

Applicants note that the above amendments were entered solely to expedite allowance of what is believed to be allowable subject matter. Applicants reserve the right to file a continuation application directed to the subject matter of the previously presented claims.

For the convenience of the Examiner, a conformed copy of the now pending claims is attached to this response.

Rejection Under 35 U.S.C. §112, first paragraph

Claims 19-28 stand finally rejected under 35 U.S.C. §112, first paragraph, as allegedly non-enabled by the specification. For the following reasons, this rejection is obviated-in-part and traversed-in-part.

Initially, this rejection is obviated-in-part because, as now amended, Claim 19 no longer references Alzheimer's disease, senile dementia and Parkinson's disease. Hence, the rejection predicated on the allegation of non-enablement due to the fact that there is no known cure for each of these diseases is no longer germane.

This rejection is further obviated-in-part because the art has established that the process of this invention does, in fact, inhibit apoptotic changes in the hippocampal cells of a mammalian (rats) brain. See, for example, Nolan, et al., [1] a copy of which is attached as Appendix A.[2] This data establishes an art recognized correlation between the claimed subject matter and *in vivo* data supporting such matter.

Secondly, this rejection is traversed-in-part because the allegation that there is no known cure for the treatment Alzheimer's disease, senile dementia and Parkinson's disease is not germane to the claimed invention. As noted during the interview, Applicants are not claiming a cure to any of such diseases but rather the claimed methods are directed to alleviating or

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[1] Nolan, Minogue, Vereker, Bolton, Campbell and Lynch, *Attenuation of LPS-Induced Changes in Synaptic Activity in Rat Hippocampus by Vasogen's Immune Modulation Therapy,* NeuroImmunoModulation, 2002-03;10:40-46

[2] The work done by this reference was supported by Vasogen, Inc. owner of Vasogen Ireland Ltd, a co-assignee of this application.

protecting against the symptoms of neurological diseases mediated by accelerated apoptosis. Any underlying etiology is not addressed by such claims.

This rejection is further traversed-in-part because the USPTO has failed to present a *prima facie* case for the instant enablement rejection, and that it must be withdrawn as a matter of law.

According to *In re Marzocchi and Horton*, "[t]he first paragraph of §112 requires nothing more than objective enablement. How such a teaching is set forth, either by the use of illustrative examples or by broad terminology, is of no importance." 169 USPQ 367, 369 (1971). The court in *Marzocchi* further stated that:

> [a]s a matter of Patent Office practice, then, a specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented *must* be taken as in compliance with the enabling requirement of the first paragraph of § 112 *unless* there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support. Assuming that sufficient reason for such doubt does exist, a rejection for failure to teach how to make and/or use will be proper on that basis; such a rejection can be overcome by suitable proofs indicating that the teaching contained in the specification is truly enabling."
> *Id.* (emphasis in original).

While the court in *Marzocchi* acknowledged that a statement contrary to "generally accepted scientific principles" could provide the basis for reasonable doubt with respect to whether an Applicant's disclosure is enabling, "it is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain *why* it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement." *Id.* at 370 (emphasis in original).

In the instant case, the Patent Office has failed to conform to Patent Office practice as set forth in *Marzocchi*. The outstanding enablement rejection was first raised in the Office Action of February 19, 2003 at page 4, which stated that:

> [t]here is no support in the instant specification for treating a neurological disorder using the claimed method. The specification fails to provide data and/or other evidence in which to enable the invention [sic]. Without such data in an art as unpredictable as biotechnology one of ordinary skill in the art would have no way of knowing if the claimed method would be able to perform the claimed method.

The Office Action of September 8, 2003 at page 2 stated that:

> [t]he level of enablement to treat (alleviate)/cure (protect against) [the diseases recited in the claims] is quite high. Applicant's claims are drawn to a method which [sic] is very unbelievable on its face. This is due to the fact that there is no cure for these conditions. Thus, without conclusive evidence to show that these conditions have been cured, the invention is not enabled by the instant disclosure.

The above two quotations represent the entirety of the "evidence or reasoning" used by the USPTO to support the rejection of claims 19-28 under 35 U.S.C. § 112, first paragraph, for alleged lack of enablement. Yet these statements are merely conclusory, unsupported by objective evidence, and failing to articulate the basis for the enablement rejection.

Applicants submit that the invention is fully enabled, *inter alia*, because the specification provides experimental data showing that the invention pre-conditions cells against cell death via apoptosis.

The court in *Marzocchi* specifically stated that illustrative examples can provide enablement for subject matter beyond the literal scope of the examples. *Id.* at 369. In *Marzocchi*, the specification provided illustrative examples of certain polyethyleneamines useful for enhancing adhesion. *Id.* at 370. The court held that the specification enabled, by example, the use of any polyethyleneamines, and that one skilled in the art would be able to identify polyethyleneamines that would not be useful for practicing the invention as a result of particular chemical properties.

Apoptosis is a well-studied cellular process that appears to be highly conserved among eukaryotic cells, and certainly mammalian neural cells. Once apoptotic pathways are triggered, the biological events that ultimately lead to cell death have been extensively studied and are becoming increasingly well understood. Thus the ability of the instant invention to decrease the

rate of apoptosis as the result of ischemia (as described in the Examples), is highly relevant to the treatment of neurological diseases that result from apoptotic cell death.

Importantly, the instant claims are drawn to methods for treating neurological disorders, which methods comprises pre-conditioning cells against apoptosis. The specification provides ample experimental data showing that the instant invention reduces apoptosis. Because apoptosis, once initiated, proceeds via a canonical series of intracellular events, methods of inhibiting apoptosis need not be dependent on or specific to upstream events that initiate apoptosis. In the instant case, the fact that the Examples relate to apoptosis in the context of ischemia, rather than neurodegeneration, should in no way limit the scope of the invention. The diseases that are currently recited in the claims are known to be associated with apoptosis. The invention clearly inhibits apoptosis. One skilled in the art would, therefore, expect the instant invention to be useful in "alleviating or protecting against the symptoms of a neurological medical disorder involving accelerated rates of apoptosis..." Claim 19.

Based on the above arguments, Applicants submit that the illustrative examples disclosed in the instant specification provide at least as much enablement for the full scope of Applicants' claims as in case of *Marzocchi*. The court in *Marzocchi* recognized that some polyethyleneamines would *not* be useful for enhancing adhesion but that identifying these molecules would be within the level of ordinary skill in the art. *Id.* at 370. In contrast, apoptosis is a biological pathway that is highly conserved among mammalian cells.[3] One skilled in the art could reasonably argue that apoptosis was a more conserved biological phenomenon among mammalian cells that adhesive enhancing properties among the plethora of conceivable polyethyleneamines.

In addition, the current lack of treatments for the neurological diseases previously recited in the claims (Office Action of September 8, 2003 at page 2) does not provide an independent or supporting basis for the instant enablement rejection. First, it is axiomatic that inventions solve problems for which there were previously no solutions. It would be absurd to reject a new

[3] With the obvious exception of anucleated cells such as erythrocytes and thrombocytes.

treatment for neurological disorders on the basis that there was no prior way to treat the disorders. Second, the instant invention affects apoptosis, which occurs as a consequence of genetic or environmental factors that lead to the diseases recited in the claims. It is irrelevant whether these diseases, *per se*, are actually treated by the instant invention or able to be treated by any means. Pre-conditioning cells against apoptosis will logically reduce the amount of cell death that would normally result from a neurological disorder that cause cells to become apoptotic. The instant invention therefore enables the full scope of the claims, drawn to "alleviating or protecting against the symptoms of a neurological medical disorder involving accelerated rates of apoptosis..." Claim 19.

Accordingly, Applicants submit that the Patent Office has entirely failed to explain, in the Office Actions of February 19, 2003 or September 9, 2003, "*why* it doubts the truth or accuracy of any statement in [the] supporting disclosure and [failed] to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement[s]." *Marzocchi,* 169 USPQ at 370 (emphasis in original). For at least these reasons, the enablement rejection must be withdrawn.

In view of the above, withdrawal of this rejection is requested.

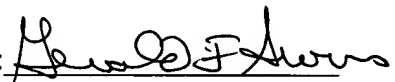
Applicants submit that the instant application is fully in condition for allowance. Early notice to that effect is earnestly solicited.

Notwithstanding the above and in order to avoid unintended abandonment of this application, a Notice of Appeal is enclosed herewith.

In the unlikely event that the transmittal letter is separated from this document and the Patent Office determines that an extension and/or other relief is required, Applicant(s) petition(s) for any required relief including extensions of time and authorizes the Assistant Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to **Deposit Account No. 50-2859** referencing docket no. 559082000800.

Respectfully submitted,

SWISS LAW GROUP

By: 
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PENDING CLAIMS

19. A method for alleviating or protecting against the symptoms of a neurological disorder involving accelerated rates of apoptosis in a mammalian body, which method comprises (a) selecting a patient having or suspected of having a neurological medical disorder mediated by accelerated rates of apoptosis; (b) reacting an aliquot of blood from the mammalian body *ex vivo* with at least one stressor selected from the group consisting of a temperature above or below body temperature, ultraviolet light and an oxidative environment; and (c) administering the aliquot of blood treated in step (b) to the mammalian body; thereby reducing the rate of or susceptibility to apoptosis of tissues and organs.

20. The method of claim 19 wherein the aliquot of blood has a volume from about 0.1 - 100 ml.

21. The method of claim 20 wherein said at least one stressor is a temperature in the range from about -5° to 55°C.

22. The method of claim 20 wherein said at least one stressor is a temperature in the range of from about 40° to 50°C.

23. The method of claim 20 wherein said at least one stressor is an oxidative environment comprising a mixture of ozone and medical grade oxygen, bubbled through the blood aliquot.

24. The method of claim 23 wherein the gaseous mixture has an ozone content of from about 10-100 μg per ml.

25. The method of claim 20 wherein said at least one stressor is ultraviolet light in the UV-C band wavelength.

26. The method of claim 20 wherein all three stressors are applied to the aliquot simultaneously.

27. The method of claim 26 wherein said stressors are applied for a period of time from 0.5 to 60 minutes.

28. The method of claim 27 wherein the time is from about 2 to 5 minutes.

Appendix A

Original Paper

NeuroImmunoModulation

Neuroimmunomodulation 2002–03;10:40–46

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Attenuation of LPS-Induced Changes in Synaptic Activity in Rat Hippocampus by Vasogen's Immune Modulation Therapy

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Key Words

Long-term potentiation · Hippocampus · IL-10 · IL-1β · Lipopolysaccharide · Vasogen's IMT

Abstract

Systemic injection of lipopolysaccharide (LPS) blocks the expression of long-term potentiation in the hippocampus of the rat. This is coupled with increased IL-1β concentration and c-Jun NH_2-terminal kinase activity, as well as an increase in the number of cells displaying apoptotic characteristics in the hippocampus. Vasogen's Immune Modulation Therapy (IMT) is a procedure involving intramuscular administration of syngeneic blood which has been exposed ex vivo to elevated temperature, oxidation and ultraviolet light. We report that Vasogen's IMT significantly abrogates these LPS-induced effects with a concomitant increase in the concentration of the anti-inflammatory cytokine IL-10. These data suggest that Vasogen's IMT may play a protective role against the deleterious effects of immune insults in the brain.

Introduction

Systemic administration of lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria, provokes activation of the immune system by inducing an increase in pro-inflammatory cytokines such as IL-1β. As well as its effects on the peripheral immune system, LPS is now known to be responsible for stimulating changes in the central nervous system, affecting processes such as thermoregulation, sleep and appetite [1]. One example of a neuronal deficit induced by LPS and IL-1β is the impairment of long-term potentiation (LTP) in the hippocampus [2, 3]. LTP is a form of synaptic plasticity and has been proposed as a biological substrate for learning and memory [4]. The inhibitory effects of both IL-1β and LPS on LTP have been linked with an increase in activity of the stress-activated protein kinase c-Jun NH_2-terminal kinase (JNK) [2]. Activation of JNK has been identified as instrumental in bringing about cell function deterioration and, ultimately, cell death [5, 6].

IL-10 is one of a number of cytokines secreted by the T helper 2 (Th2) subclass of lymphocytes and is known for its anti-inflammatory effects. Anti-inflammatory cytokines, such as IL-10, have been reported to prevent IL-1β-induced changes [7, 8], thus inhibiting pro-inflammatory responses. It has been demonstrated that Vasogen's Immune Modulation Therapy (IMT), which involves intramuscular administration of syngeneic blood following ex vivo treatment with elevated temperature, oxidation

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and ultraviolet light [9], appears to mediate a Th1 to Th2 immunodeviation in patients suffering from scleroderma, a Th1-mediated disease [10]. There is evidence that Vasogen's IMT suppresses contact hypersensitivity [11] and reduces the progression of atherosclerosis in low-density lipoprotein receptor-deficient mice [12], suggesting a possible anti-inflammatory activity. The proposed role of Vasogen's IMT in interfering with pro-inflammatory responses prompted an investigation into its effect on LPS-induced neuroinflammation. In this study we assessed changes in LTP, IL-1β concentration, JNK activity and evaluated TUNEL staining for fragmented DNA, a characteristic of apoptotic cells.

Materials and Methods

Animals and Treatment Protocol

Male Wistar rats (300–350 g; BioResources Unit, Trinity College Dublin, Ireland) were used in these experiments. Animals were housed in groups of 4 under a 12-hour light/dark schedule with free access to food and water. Ambient temperature was controlled between 22 and 23°C and rats were maintained under veterinary supervision.

Whole blood (11 ml) was obtained from donor rats by cardiac puncture and added to 2.2 ml of 3.13% sodium citrate solution, of which 1.2 ml was removed and used for sham treatment. For Vasogen's IMT, the remaining 12 ml of anticoagulated blood were transferred to a single-use blood container (VC7002, Vasogen Inc, Toronto, Canada) and exposed to a combination of controlled physiochemical stress factors in a medical device (VC7001, Vasogen Inc.). The medical device executed an automated procedure during which the temperature of the blood was first raised (to a nominal temperature of 42.5°C) over a period of 6–8 min. A gas mixture of ozone in medical oxygen (nominal concentration 14.5 μg/ml) was then applied to the blood (nominal flow rate 240 ml/min) for 3 min. During this time the blood was exposed to UVC light (maximum emission spectrum at 254 nm). Finally, the treated blood was allowed to settle for at least 7 min prior to removal from the blood container. Rats were treated by intramuscular injection of 150 μl of processed blood or untreated blood (sham treatment). Injections were administered 14 days, 13 days and 1 day before LPS or saline challenge. Rats were divided into 4 treatment groups, which will be referred to as sham-saline, sham-LPS, IMT-saline, IMT-LPS.

Induction of LTP in Perforant Path-Granule Cell Synapses in vivo

LTP was induced as described previously [13]. Rats were anaesthetised by an intraperitoneal urethane injection (1.5 g/kg), subsequently received either LPS (100 μg/kg) or saline intraperitoneally and were monitored for 3 h. Rats were then placed in a head holder in a stereotaxic frame. A window of skull was removed to allow placement of recording and stimulating electrodes in the molecular layer of the dentate gyrus (2.5 mm lateral and 3.9 mm posterior to bregma) and perforant path (angular bundle, 4.4 mm lateral to lambda), respectively. The depth of the electrodes was adjusted to obtain maximal responses in the cell body region and, after an initial period to allow baseline responses to stabilise, test shocks were delivered to the perforant path at the rate of 1/30 s. Responses were recorded for 10 min prior to and 40 min following tetanic stimulation (3 trains of stimuli; 250 Hz for 200 ms; intertrain interval 30 s). At the end of the electrophysiological recording period, rats were killed by decapitation, the hippocampus was removed, dissected on ice and cross-chopped into slices (350 μm × 350 μm), using a McIlwain tissue chopper. The time needed to prepare slices from the time of death was 2.5–3.5 min. All samples were frozen separately in 1 ml Krebs solution (composition, in m*M*: NaCl 136, KCl 2.54, KH_2PO_4 1.18, $MgSO_4 \cdot 7\,H_2O$ 1.18, $NaHCO_3$ 16, glucose 10, $CaCl_2$ 1.13) containing 10% dimethylsulphoxide [14]. For analysis, thawed slices of tissue were rinsed 3 times in fresh ice-cold Krebs solution and homogenized in ice-cold Krebs solution.

Analysis of IL-1β Concentration

IL-1β concentration in hippocampal homogenate was analysed by ELISA (R & D Systems, UK). Antibody-coated (100 μl; 1.0 μg/ml final concentration, diluted in phosphate buffered saline (PBS), pH 7.3; goat anti-rat IL-1β antibody), 96-well plates were incubated overnight at room temperature, washed several times with PBS containing 0.05% Tween 20 and blocked for 1 h at room temperature with 300 μl blocking buffer (PBS, pH 7.3, containing 5% sucrose, 1% bovine serum albumin (BSA), and 0.05% NaN_3). After several washes, plates were incubated with IL-1β standards (100 μl; 0–1,000 pg/ml in PBS containing 1% BSA) or samples (homogenised in Krebs solution containing 2 m*M* $CaCl_2$) for 2 h at room temperature. Samples were incubated with secondary antibody (100 μl; final concentration 350 ng/ml in PBS containing 1% BSA and 2% normal goat serum; biotinylated goat anti-rat IL-1β antibody) for 2 h at room temperature, washed and incubated in detection agent (100 μl; horseradish peroxidase conjugated streptavidin; 1:200 dilution in PBS containing 1% BSA) for 20 min at room temperature. Substrate solution (100 μl; 1:1 mixture of H_2O_2 and tetramethylbenzidine) was added, samples were incubated at room temperature in the dark for 1 h, the reaction was stopped using 50 μl 1 M H_2SO_4. Absorbance was read at 450 nm, values were corrected for protein [15] and expressed as pg/mg protein.

Analysis of IL-10 Concentration

A commercially available ELISA (Biosource International Inc., USA) was used to analyse IL-10 concentration in the hippocampus. Samples were homogenised in Iscove's culture medium containing 5% fetal bovine serum and a cocktail of enzyme inhibitors (100 m*M* amino-n-caproic acid; 10 m*M* Na_2EDTA; 5 m*M* benzamidine HCl; 0.2 m*M* phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 10,000 rpm at 4°C for 10 min, the supernatant was removed and analysed for IL-10 using ELISA. Optical densities were determined at 450/630 nm dual wavelength mode using a multi-well plate reader; values were corrected for protein [15] and expressed as pg/mg protein.

Analysis of JNK Activity

The activity of JNK was analysed in homogenate prepared from frozen hippocampal slices. In a separate experiment, activity of the kinase was assessed in freshly prepared hippocampal synaptosomes obtained from untreated rats. These samples had been pre-treated for 20 min in the absence and presence of IL-1β (1 ng/ml) and vasoactive intestinal peptide (VIP; 1 μ*M*), a proven JNK inhibitor [16]. In all experiments, samples were analysed for protein [15], and diluted to

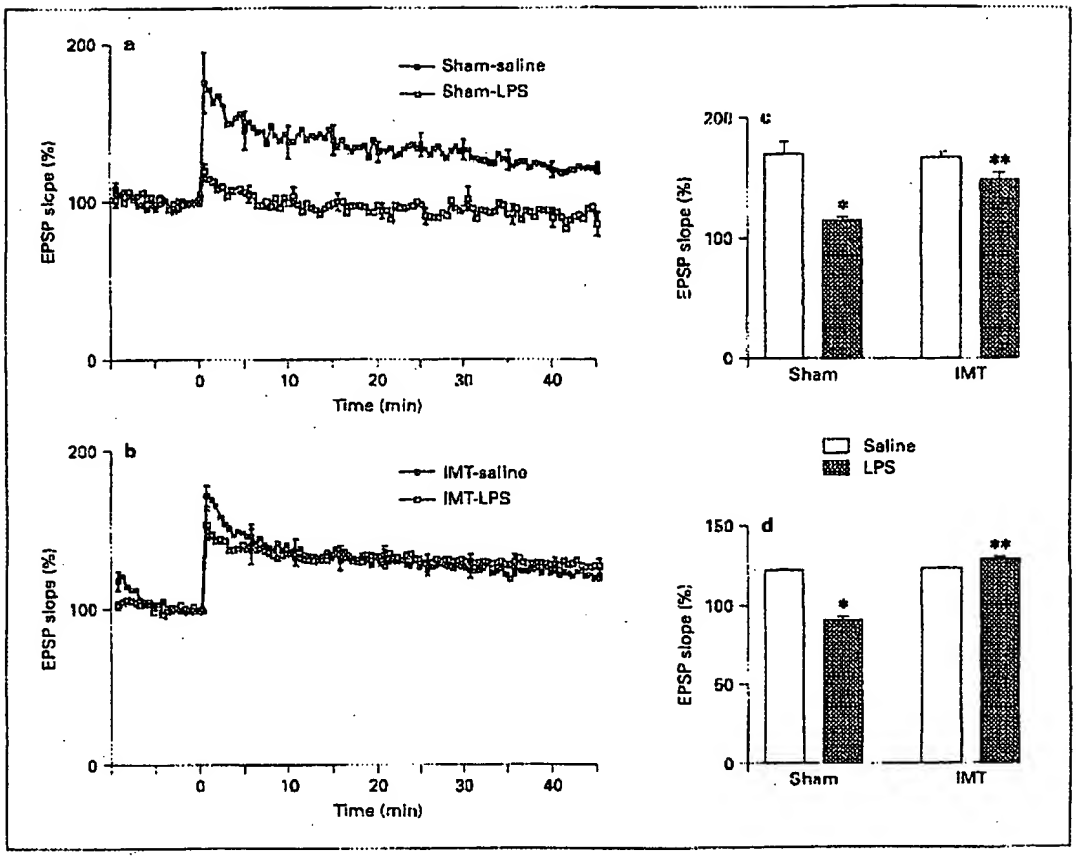


Fig. 1. Intraperitoneal injection of LPS inhibits LTP in perforant path-granule cell synapses (**a**). This inhibitory effect is prevented by pre-treatment with Vasogen's IMT, which exerted no significant effect in saline-treated rats (**b**). The data presented are means of 7–8 observations in each treatment group. Data are expressed as mean percentage change in EPSP slope every 30 s, normalised with respect to the mean value in the 5 min immediately prior to tetanic stimulation. SEM are included for every 10th response. Analysis of the mean values in the 2 min immediately following tetanic stimulation (**c**) and in the last 5 min of the experiment (**d**) indicate that population EPSP slope was significantly decreased in the sham-LPS group (* $p < 0.01$ vs. sham-saline; ANOVA), but that Vasogen's IMT significantly reversed this effect (** $p < 0.01$ vs. sham-LPS; ANOVA). These values are means ± SEM of 7–8 observations in each case.

equalise for protein concentration. These samples (10 µl, 1 mg/ml) were added to 10 µl sample buffer (Tris-HCl 0.5 m*M*, pH 6.8; glycerol 10%; SDS 10%; β-mercaptoethanol 5%; bromophenol blue 0.05% w/v), boiled for 5 min and loaded onto gels (10% SDS). Proteins were separated by application of a 30-mA constant current for 25–30 min, transferred onto nitrocellulose strips (225 mA for 75 min) and immunoblotted by incubation with an antibody that specifically targets phosphorylated JNK [Santa Cruz Biotechnology, USA; 1:200 in Tris-buffered saline Tween (0.1% Tween-20) containing 1% BSA] for 2 h at room temperature. Nitrocellulose strips were washed and incubated for 2 h at room temperature with secondary antibody (peroxidase-linked anti-mouse IgG; 1:300 dilution; Sigma, UK). Visualization was achieved using SuperSignal West Dura Extended Duration Substrate (Pierce, USA). Immunoblots were immersed in substrate for 5 min and subsequently exposed to film for 1 s. Film was processed using a Fuji X-ray processor, and quantification of protein bands was achieved by densitometric analysis using two software packages: Grab It (Grab It Annotating Grabber 2.04.7, Synotics; UVP Ltd., UK) and Gelworks (Gelworks ID, Version 2.51; UVP Ltd) for photography and densitometry, respectively. Gelworks provides a single value (in arbitrary units), representing the density of each blot; the values presented here are means of data generated from at least 4 separate experiments.

TUNEL Staining

Dissociated cells were prepared by enzymatic and mechanical digestion of fresh hippocampal slices. Slices were incubated with collagenase (0.125%; Sigma) in PBS for 30 min at room temperature,

washed with PBS to terminate collagenase digestion, and then gently triturated with a glass Pasteur pipette before passing through a nylon mesh filter to remove tissue clumps. Cells were then cytospun onto glass microscope slides, fixed with methanol and stored until use.

TUNEL (Terminal deoxynucleotidyl Transferase (TdT)-mediated dUTP Nick-End Labelling) staining, which identifies nuclei with fragmented DNA (a characteristic of apoptotic cells), was performed according to the manufacturer's (Promega, USA) instructions. Briefly, fixed cytospun cells were washed and permeabilised with 0.2% Triton in PBS. Cells were equilibrated in buffer (200 m*M* potassium cacodylate (pH 6.6 at 25°C), 25 m*M* Tris-HCl (pH 6.6 at 25°C), 0.2 m*M* DTT, 0.25 mg/ml BSA, 2.5 m*M* $CoCl_2$) for 5 min at room temperature and incubated in TdT reaction mixture (30 µl; 98 µl equilibration buffer, 1 µl biotinylated nucleotide mix, 1 µl TdT enzyme) at 37°C for 1h. The reaction was terminated by adding 100 µl 2 × SCC (1:10; 2 × SCC:deionised water), endogenous peroxidases were blocked by incubation with H_2O_2 (100 µl; 0.3% in PBS) for 5 min at room temperature, and washed cells were incubated for 30 min at room temperature in streptavidin HRP solution (100 µl; 1:500 in PBS) to allow binding to the biotinylated nucleotides. Diaminobenzidine solution was added to washed cells, and the incubation proceeded for 10 min at room temperature. Cells were washed with deionised water, dehydrated through graded ethanol, cleared with xylene and then slides were mounted in DPX mounting medium and coverslipped. TUNEL-positive cells were expressed as a percentage of the total.

Statistical Analysis

Data were analysed, as appropriate, using either Student's t test for independent means, or a one-way analysis of variance (ANOVA) followed by post hoc Student Newman-Keuls test to determine which conditions were significantly different from each other. Data are expressed as means with standard errors and deemed statistically significant when $p < 0.05$.

Results

In the sham-LPS group, tetanic stimulation delivered to the perforant path 3 h after intraperitoneal injection of LPS resulted in an increase in the mean slope of the population excitatory post-synaptic potential (EPSP) recorded in cell bodies of the granule cells. The mean percentage change (± SEM) in the 2 min immediately following tetanic stimulation compared with 5 min immediately before tetanic stimulation was 114.49 ± 2.79. This was not maintained, however, so that the mean percentage change in population EPSP slope in the last 5 min of the experiment was 90.32 ± 2.42 in the sham-LPS group. The corresponding values in the sham-saline groups of rats were 170.15 ± 10.16 and 121.28 ± 1.20, respectively (fig. 1a).

The LPS-induced inhibition of LTP was overcome by pre-treatment with Vasogen's IMT. The mean percentage change in population EPSP slope (mean ± SEM) in the 2 min immediately after tetanic stimulation was 147.44 ± 5.84 in the IMT-LPS group compared with 166.85 ± 4.54 in the IMT-saline group. In the last 5 min of the experiment, the values were 128.07 ± 1.46 for the IMT-LPS group and 121.96 ± 0.85 for the IMT-saline group (n = 7–8; fig. 1b). The values in the 2 min immediately after tetanic stimulation and in the last 5 min of the experiment were similar in sham-saline and IMT-saline groups ($p > 0.05$). Statistical analysis of the data (fig. 1c, d) revealed that both the early and late phases of LTP were markedly reduced by LPS administration in sham-treated rats ($p < 0.01$ in both cases; ANOVA), and that pre-treatment with Vasogen's IMT significantly attenuated the effect of LPS ($p < 0.01$ in both cases; ANOVA).

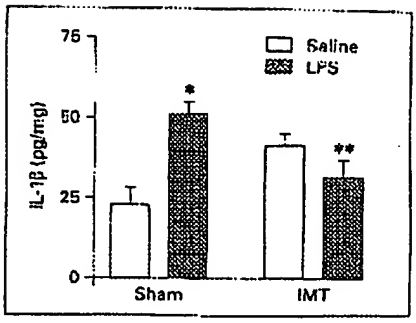


Fig. 2. LPS stimulated a significant increase in the concentration of hippocampal IL-1β in sham-treated animals (* $p < 0.01$ vs. sham-saline; ANOVA). This effect was significantly abrogated in animals pre-treated with Vasogen's IMT (** $p < 0.05$ vs. sham-LPS; ANOVA). Data are expressed as means ± SEM, n = 7–8.

Figure 2 shows that IL-1β concentration in the hippocampus was significantly increased in the sham-LPS group compared to the sham-saline group ($p < 0.01$; ANOVA); this increase was significantly attenuated by pre-treatment with Vasogen's IMT ($p < 0.05$; ANOVA).

The LPS-induced changes in IL-1β and LTP in sham-LPS rats were associated with an increase in JNK activity in the hippocampus (fig. 3a). In animals treated with Vasogen's IMT, however, these differences were coupled with an attenuated LPS-induced increase in JNK ($p < 0.05$; ANOVA; fig. 3a). Thus JNK activation was increased in the hippocampi of sham-treated animals challenged with LPS [compare lanes 1 (sham-saline) and 2 (sham-LPS)]. This effect was attenuated in rats treated with Vasogen's IMT [compare lanes 2 (sham-LPS) and 4 (IMT-LPS)], which exerted no effect if given on its own [lane 3 (IMT-saline)]. Mean data obtained from densitometric analysis revealed that LPS challenge significantly increased JNK activation in sham-treated animals by

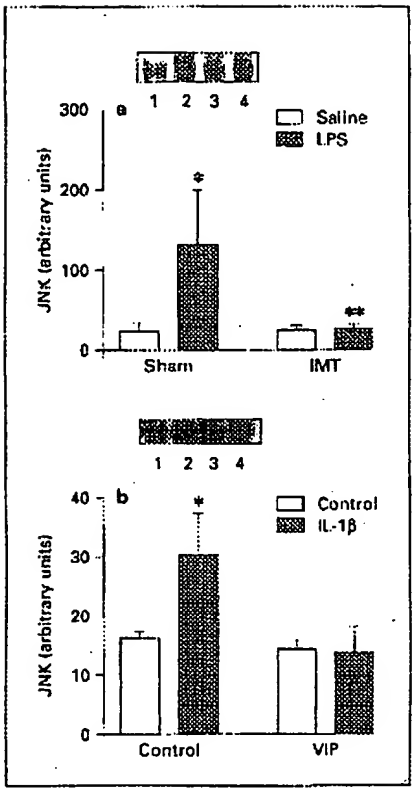


Fig. 3. The LPS-induced increase in JNK activity is blocked by pre-treatment with Vasogen's IMT (**a**). LPS induces a significant increase in JNK activity in the hippocampi of sham-treated rats, as indicated by an increase in the phosphorylated form of JNK (JNK-1 isoform 46 kD; * $p < 0.05$ vs. sham-saline; ANOVA). Analysis of the mean data obtained from densitometric analysis indicated that Vasogen's IMT significantly reduced this effect of LPS. (** $p < 0.05$ vs. sham-LPS; ANOVA). Sample immunoblots indicate the stimulatory effects of LPS (lane 2) on JNK activity in the absence of Vasogen's IMT (compare lanes 1 and 2) and the inhibition of this effect after pre-treatment with Vasogen's IMT (compare lanes 2 and 4). Data are expressed as means ± SEM, n = 7–8. In vitro, VIP blocks IL-1β-induced increase in JNK activity (**b**). IL-1β induces a significant increase in JNK activity (* $p < 0.05$ vs. control; ANOVA; compare lanes 1 and 2), but this effect is blocked by co-incubation with VIP (compare lanes 2 and 4). Data are expressed as means ± SEM, n = 6.

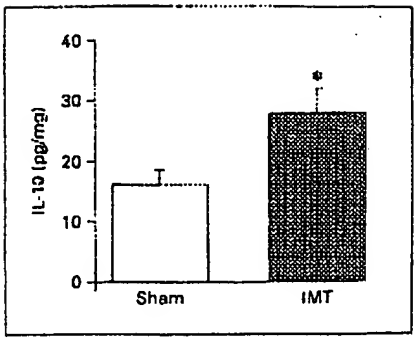


Fig. 4. The concentration of anti-inflammatory cytokine IL-10 was significantly increased in hippocampal tissue as a result of treatment with Vasogen's IMT (* $p < 0.05$ vs. sham; Student's t test for independent means). Data are expressed as means ± SEM, n = 7–8.

LPS challenge ($p < 0.05$; ANOVA), whereas no parallel effect of LPS on JNK activation was observed in tissue prepared from rats treated with Vasogen's IMT.

When JNK activity was analysed after pre-incubating freshly prepared hippocampal synaptosomes from untreated rats in the absence and presence of IL-1β and the non-specific JNK inhibitor VIP, a VIP-associated attenuation of IL-1β-induced activity was observed (fig. 3b). In vitro, IL-1β induced a significant increase in JNK activity ($p < 0.05$; ANOVA; compare lanes 1 and 2), but this effect was blocked by co-incubation with VIP (compare lanes 2 and 4).

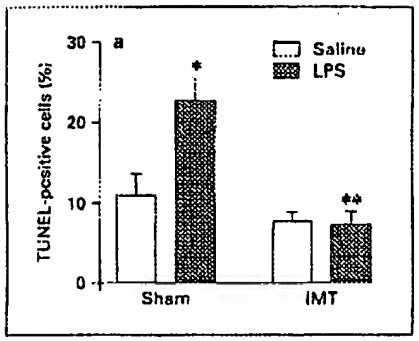
Analysis of IL-10 in the rat hippocampus revealed that Vasogen's IMT was associated with a significant increase in IL-10 relative to sham treatment ($p < 0.05$; Student's t test for independent means; fig. 4).

Figure 5a demonstrates that the percentage of dissociated cells prepared from fresh hippocampal tissue staining positive for TUNEL was significantly increased in the sham-LPS group compared with the sham-saline group ($p < 0.01$; ANOVA). Animals treated with Vasogen's IMT did not display this degenerative effect of LPS ($p < 0.01$; ANOVA). A representative image of TUNEL-positive cells shows an increased number of apoptotic cells after LPS injection, as evidenced by increased number of cells displaying dark brown stained nuclei, i.e. TUNEL-positive cells (fig. 5bii). This contrasts with cells prepared from hippocampi of sham-saline (fig. 5bi) and IMT-saline rats (fig. 5biii). Figure 5biv shows a reduction in the number of cells displaying TUNEL-positive staining in the IMT-LPS group.

Discussion

The objective of this study was to investigate the possibility that pre-treating rats with Vasogen's IMT may block the LPS-induced inhibitory effects on synaptic plasticity in the hippocampus. Accordingly, the data demonstrate that the LPS-induced inhibition of LTP in perforant path-granule cell synapses was abrogated by pre-treatment with Vasogen's IMT.

Systemic injection of LPS in sham-treated animals induced an increase of IL-1β in the hippocampus, a find-



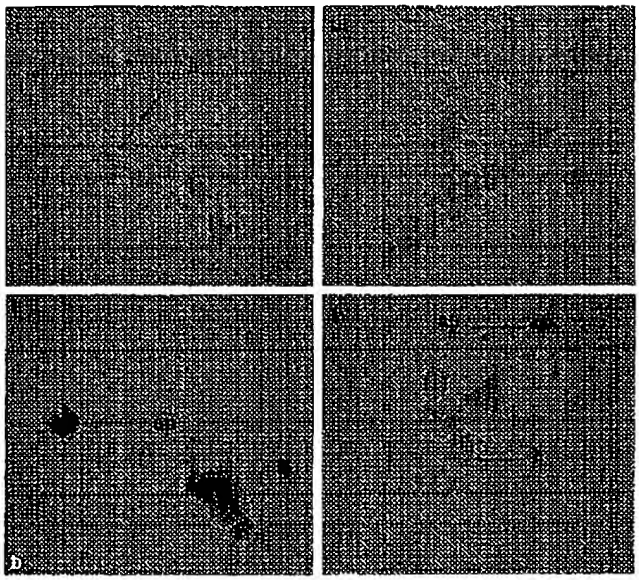


Fig. 5. The LPS-induced apoptotic changes in hippocampal cells were inhibited by pre-treatment with Vasogen's IMT. Cytospun cells were prepared from the hippocampi of rats and the mean data, obtained by counting 200 cells on each coverslip, show a significant increase in the percentage of apoptotic cells in the sham-LPS group compared with sham-saline rats (* $p < 0.01$ vs. sham-saline; ANOVA). This percentage increase is reversed by pre-treatment with Vasogen's IMT (** $p < 0.01$ vs. sham-LPS; ANOVA; **a**). Data are expressed as mean ± SEM. n = 5. Representative image TUNEL staining displays healthy (h) and apoptotic (ap) cells (**b**). There is an increased number of dark brown stained cells prepared from hippocampi of rats injected with LPS (ii) compared with cells prepared from saline-injected control rats (i), and rats treated with IMT only (iii). Pre-treatment with IMT reversed the effects of LPS with fewer cells displaying brown staining (iv). Scale bar is 20 μm.

ing that supports earlier reports [2, 17]. It has been proposed that LPS may inhibit LTP in perforant path-granule cell synapses as a consequence of an LPS-induced increase in IL-1β concentration in the hippocampus [2]. Indeed, it has previously been reported that intracerebroventricular injection of IL-1β inhibits LTP in perforant path-granule cell synapses in vivo [3, 8], and that IL-1β attenuates LTP in dentate gyrus in vitro [18]. Pre-treatment with Vasogen's IMT prevented the LPS-stimulated increase in hippocampal IL-1β concentration, as well as the LPS-induced inhibition of LTP.

Data from this laboratory have demonstrated a stimulatory effect of both LPS and IL-1β on JNK activation [2, 19]. As a consequence of the preventative effect of Vasogen's IMT on the LPS-induced increase in IL-1β concentration, the stimulatory effect of LPS on JNK activity was also attenuated. Here we also report that co-incubation of synaptosomes in the presence of IL-1β and the non-specific JNK inhibitor VIP blocks the IL-1β-induced increased activation of JNK. Thus it seems reasonable to propose that Vasogen's IMT may exert its protective effect on synaptic function by acting to prevent this LPS-induced signalling event.

Another consequence of peripheral administration of LPS is neuronal degeneration, as demonstrated by increased numbers of cells whose nuclei display fragmented DNA, a characteristic associated with apoptosis [2, 6, 20]. Accordingly, we have demonstrated a significant increase in the percentage of TUNEL-positive cells in the hippocampus as a result of LPS administration in sham-treated animals. It is possible that the impairment in LTP due to LPS injection in sham-treated animals may be due to degenerative changes in hippocampal cells. Thus it is not unreasonable to suggest that the prevention of LPS-induced inhibition of LTP by Vasogen's IMT is paralleled by a prevention of LPS-induced cell death. The present evidence, which shows an abrogation of LPS-induced increase in TUNEL-positive hippocampal cells by Vasogen's IMT, supports this proposal. Concurrent with this finding is the observation that pre-treatment with Vasogen's IMT reduces apoptosis after acute renal ischemia/reperfusion injury in dogs, as estimated by the reduction in mitochondrial membrane potential [21].

It has recently been shown that intracerebroventricular administration of the anti-inflammatory cytokine IL-10 in vivo reverses IL-1β-induced impairment of LTP and JNK activation in the hippocampus [8]. Injection of IL-10 has also been shown to reduce LPS-induced fever [22] and the behavioural effects induced by LPS [23]. In addition, IL-10 has been shown to confer protection against oligo-

dendroglial death evoked by LPS/IFN-γ in vitro [24]. In the present study, pre-treatment with Vasogen's IMT caused a significant increase in IL-10 concentration in the hippocampus. This finding is consistent with the observation that administration of Vasogen's IMT inhibits Th1-mediated contact hypersensitivity in mice [11], and the level of suppression was comparable to that seen with animal models using IL-10 [25]. Therefore, it is possible that in the present experimental paradigm, Vasogen's IMT may exert its beneficial effects by suppressing the Th1 response with a concomitant release of anti-inflammatory IL-10, as suggested by the obstruction of LPS-induced pro-inflammatory effects. Whether the IMT-induced central decrease in IL-1β and the increase in IL-10 observed in the study are due to a peripheral decrease in IL-1β and increase in IL-10 is as yet unknown. Current information on the mechanism of immune molecule trafficking across the blood-brain barrier is limited. However, some signals that regulate immune cell traffic include intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on activated brain endothelia, and their counter-receptors LFA-1 and VLA-4 on immune cells. It remains to be investigated whether peripheral administration of Vasogen's IMT induces its central effects by activation of these mediators.

Although the exact mechanism of action of Vasogen's IMT remains to be elucidated, there is clear evidence from the data presented in this study that pre-treatment with the therapy confers a protective effect on the organism by preventing LPS-induced impairment of synaptic function and the resultant detrimental effects in the hippocampus of the rat.

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